The *Erwinia chrysanthemi phoP-phoQ* operon plays an important role in growth at low pH, virulence and bacterial survival in plant tissue

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Summary

We have studied the role of acidic pH as a barrier for the colonization of the plant apoplast by *Erwinia chrysanthemi*. A minitransposon containing a promoterless reporter gene, *gus*, was used for random mutagenesis of the bacterial genome. An acid-sensitive mutant, named BT119, was isolated and had the following differential features with respect to the wild-type strain: (i) inability to grow at pH ≤ 5.5; (ii) decreased survival at acid pH and in plant tissues; (iii) increased susceptibility to antimicrobial peptides; (iv) decreased virulence in chicory leaves and pear fruits; (v) reduced polygalacturonase production; and (vi) reduced ability to alkalinize chicory tissues after infection. The sequence of the interrupted gene was highly similar to the *phoQ* gene, which is involved in environmental sensing in several bacteria, such as *Yersinia pseudotuberculosis*, *Erwinia carotovora*, *Salmonella typhimurium* and *Escherichia coli* and thus, this designation was used for the *E. chrysanthemi* system. This gene was induced at low Mg^{2+} concentrations and *in planta*. These results suggest that *E. chrysanthemi* PhoP-PhoQ system plays an important role in bacterial survival in plant tissues during the initial infection stages.

Introduction

Most phytopathogenic bacteria are specialists in colonizing the plant apoplast. This particular niche is nutrient-poor (Alfano and Collmer, 1996) (particularly in iron) (Expert, 1999) and it is laden with several toxic substances (either preformed or induced) that constitute an important barrier for the development of bacterial populations (Osbourn, 1996; García-Olmedo *et al.*, 1998; Segura *et al.*, 1999). A possible involvement in plant defence has been postulated for the following types of inhibitory molecules: (i) some of the pathogenesis-related proteins (Slusarenko *et al.*, 2000); (ii) a considerable variety of plant organic compounds, classified into phytoanticipins and phytoalexins (Osbourn, 1996); (iii) plant antimicrobial peptides (García-Olmedo *et al.*, 1998); and (iv) active oxygen and nitrogen species, such as hydrogen peroxide and peroxynitrite (Alamillo and García-Olmedo, 2001). The importance of plant antimicrobial substances in defence is highlighted by the fact that bacterial mutants with increased sensitivity to some of these compounds were also less virulent (Titarenko *et al.*, 1997; López-Solanilla *et al.*, 1998; 2001; El Hassouni *et al.*, 1999).

Plant apoplast is acidic, generally ranging from pH 4.5 to pH 6.5 (Grignon and Sentenac, 1991), and poses a significant barrier for the growth of pathogenic bacteria. The low pH is due to the abundance of organic acids, such as citric and malic acids, and the extrusion of protons from adjacent cells (Grignon and Sentenac, 1991). Bacterial resistance and adaptation to acidic pH has been extensively studied in the *Enterobacteriaceae*. Neutrophilic bacteria, such as *Salmonella typhimurium* and *Escherichia coli*, often encounter acid stress conditions both in nature and during infection, for example, within the stomach or inside the macrophage phagolysosomes. Acid stress is a complex phenomenon involving the combined effects of acidic pH and organic acids (Bearson *et al.*, 1998). To resist this type of stress, enteric bacteria have evolved several mechanisms, including: (i) inducible expression of amino acid decarboxylases, which consume protons as a result of their enzymatic activity (Bearson *et al.*, 1997); (ii) regulatory networks (RpoS, PhoP, Fur) that control several genes involved in acid tolerance and are activated by acidic stress (Foster and Moreno, 1999); and (iii) induction of chaperones and DNA repairing enzymes, which counteract the damage produced by acidic pH (Bearson *et al.*, 1997).

Erwinia chrysanthemi is a member of the *Enterobacteriaceae* which causes soft-rot diseases in a wide range of economically important crops (Dickey, 1979; Boccara *et al.*, 1991). The pathogenic behaviour of this bacterium

is characterized by a rapid necrosis of parenchymatous tissues and soft-rot symptoms, mainly caused by pectic enzymes that degrade the middle lamellae and the primary cell wall (Bateman and Basham, 1976; Barras *et al.*, 1994). It has been reported that when *E. chrysanthemi* infects the apoplastic space, the secreted pectic enzymes degrade cell walls, and the resulting cell lysis leads to a pH increase of the intercellular environment (Nachin and Barras, 2000). In turn, this increase enhances the overall enzymatic activity of pectate-lyases (Collmer and Keen, 1986), thus implementing a feed-back process which ultimately leads to tissue maceration (Nachin and Barras, 2000). In addition, other factors that are known to contribute to the virulence of this bacterium include: resistance to antimicrobial peptides (López-Solanilla *et al.*, 1998; 2001), iron transport functions (Sauvage and Expert, 1994; Franza *et al.*, 1999), and *hrp* genes (*h*ypersensitive *r*esponse and *p*athogenicity) which encode a type III secretion system involved in the delivery of proteins to the plant cell (Bauer *et al.*, 1994).

The object of this work was to study the role of acidic pH as a barrier for the colonization of the apoplast by *Erwinia chrysanthemi*. A Tn*5* mutant impaired in its ability to grow at pH 5.5 was isolated and found to be also affected in its virulence, resistance to antimicrobial peptides and polygalacturonase production. The sequence of the inactivated genes was similar to the PhoP/PhoQ system of several bacteria and appeared to play an important role in survival in plant tissues during the initial stages of infection.

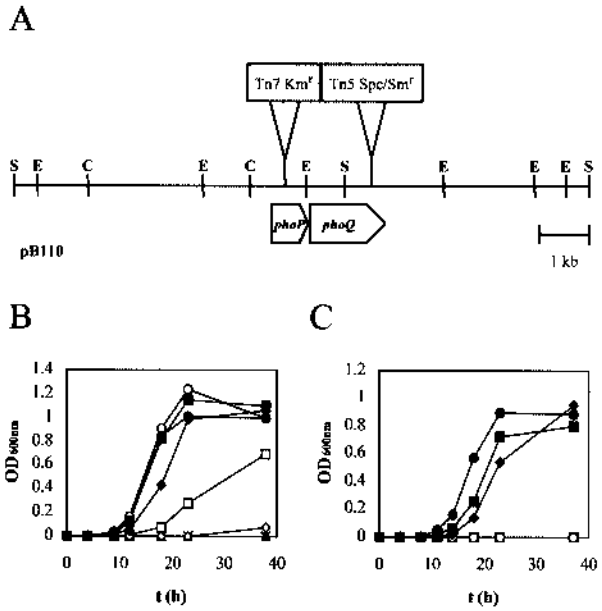

Fig. 1. A. Genetic and physical map of the insert of pB110 clone from *E. chrysanthemi*. The insertion points of Tn*5* in *phoQ* mutant and Tn*7* in *phoP* mutant are indicated. C, *Cla*I; E, *Eco*RI; S, *Sac*I. Km^r, kanamycin resistance; Spc/Sm^r, spectinomycin/streptomycin resistance.
B. Growth curves of the wild-type strain in MBMA at different pHs. Closed diamonds, pH 7.0; closed squares, pH 6.5; closed circles, pH 6.0; open circles, pH 5.5; open squares, pH 5.0; open diamonds, pH 4.5; closed triangles, pH 4.0; open triangles, pH 3.5; asterisk, pH 3.0.
C. Growth curves of BT119 strain in MBMA at different pHs. Closed diamonds, pH 7.0; closed squares, pH 6.5; closed circles, pH 6.0; open circles, pH 5.5; open squares, pH 5.0; open diamonds, pH 4.5; closed triangles, pH 4.0; open triangles, pH 3.5; asterisk, pH 3.0.

Results

Isolation and characterization of E. chrysanthemi *EC16 mutants impaired in its ability to grow at pH 5.5*

Growth curves of *E. chrysanthemi* EC16 at different pHs were determined as a preliminary step in the isolation acid sensitive mutants. This bacterium was grown in basal medium adjusted to different pH values, ranging from 3.0 to 7.0 (Fig. 1B). We finally chose pH 5.5 to screen for acid sensitive bacteria because: (i) it is known that this pH poses a significant acid stress for enteric bacteria (Bearson *et al.*, 1997); (ii) *E. chrysanthemi* EC16 grew well at this pH, suggesting the presence of acid-resistance mechanisms (this work); and (iii) this pH can be considered as 'typical' of the plant apoplast (Grignon and Sentenac, 1991).

Random mutagenesis of the bacterial genome was performed with a minitransposon containing a promoterless *gus* reporter gene (Wilson *et al.*, 1995). A total of 2500 spectinomycin/streptomycin-resistant, ampicillin-sensitive insertions were screened in modified basal medium A (MBMA) at pH 5.5, and subsequently at pH 7.0. Two mutants, unable to grow at pH 5.5, but with normal growth at pH 7.0, were isolated and one, named BT119, was selected for further studies. Genomic DNA from this mutant was obtained and a fragment containing the transposon was isolated, by using a fragment from the minitransposon as a probe. This fragment was subcloned in a pBluescript SK(–) vector, and sequenced. The sequence of the flanking DNA was found to be highly similar to the *phoQ* gene of several bacteria, using the BLAST service (http://www.ncbi.nlm.nih.gov) and thus, the same designation was used for the *E. chrysanthemi* genes.

In order to clone the complete *phoP-phoQ* operon, a λ-FIX II genomic library of *E. chrysanthemi* EC16 was constructed and probed with the previously identified *phoQ* fragment. A clone including the complete sequence (pB110) was identified, after restriction mapping, subcloning in vector pBluescript SK(–), and sequencing (Table 1 and Fig. 1A). Two open reading frames were found that were, respectively, homologous to the PhoP proteins of *Yersinia pseudotuberculosis* (83%), *Escherichia coli* (81%) and *Salmonella typhimurium* LT2 (80%), and PehR protein of *E. carotovora* (82%); and to the PhoQ proteins of *Yersinia pseudotuberculosis* (69%), *Escherichia coli*

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	*supE44 Δlac U169 (ø80 lacZM15) hsdR17 recA1endA1 gyrA96 thi-1relA1*	Hanahan (1983)
XL1-Blue MRA	*Δ(mcrA) 183 Δ(mcrCB- hsdSMR-mrr) 173 endA1supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
XL1-Blue MRA (P2)	XL1-Blue MRA (P2 lysogen)	Stratagene
S17.1-λ*pir*	294::[RP4-2 (Tc::Mu) (Km::Tn*7*)] *pro res* Δ*recA*, Tp^r, mod+, λ*pir*+	De Lorenzo and Timmis (1994)
E. chrysanthemi		
EC16	Wild-type strain	Chatterjee *et al.* (1983)
BT119	*Δ(phoQ)*::Tn*5* Spc/Sm^{r}derivative of EC16	This work
BT118	*Δ(phoP)*::Tn*7* Km^{r}derivative of EC16	This work
BT121	BT119 complemented with pB112, Spc/Sm^r, Cam^r	This work
Plasmids and phages		
pGEM® T-Easy	Amp^r	Promega
pBluescript II SK(–)	Amp^r	Stratagene
pTrueBlue-BAC2®	Cam^r	Genomics One
pCAM140	mTn*5*SS*gusA40* Spc/Sm^r Amp^r	Wilson *et al.* (1995)
pB110	pBluescript II carrying EC16 *phoP* and *phoQ* genes	This work
pB111	pB110::Tn*7* Km^r	This work
pB112	pTrueBlue-BAC2 carrying EC16 *phoP* and *phoQ* genes	This work
λ-FIX II	Phage vector	Stratagene

(62%) and *Salmonella typhimurium* LT2 (59%), and PehS protein of *E. carotovora* (76%). The *E. chrysanthemi* genes were, respectively, named *phoP* and *phoQ* (Fig. 1A). Clone pB110 was subjected to Tn*7* *in vitro* mutagenesis, and one mutagenized construction (pB111), bearing the Tn*7* transposon within the *phoP* gene, was selected (Fig. 1A, Table 1) and marker-exchanged into the *E. chrysanthemi* EC16 chromosome. Out of several Amp^S Km^R recombinants (data not shown), one mutant strain, BT118, was selected for further analysis. The *phoQ* mutant (BT119) was analysed by using the BIOLOG-Microlog System, and no difference in the utilization of carbon sources was found. This mutant and the wild-type strain had essentially the same growth rate in liquid medium (data not shown). The growth of *phoQ* mutant was checked at different pHs in MBMA, and its inability to grow below pH 6.0 was confirmed (Fig. 1C). Also, the *phoP* mutant was not able to grow at pH 5.5 (data not shown). A complemented strain (BT121) was constructed using a unique-copy vector (Table 1). Strain BT121 was able to grow in acid pH as the wild type.

The sensitivity of BT119 strain to antimicrobial peptides, derived from plants and animals, was compared with that of the wild type. Inhibition tests were performed *in vitro* with salmon protamine and wheat thionin. As shown in Fig. 2, the mutant was significantly more susceptible to both antimicrobial peptides. Because bacterial sensitivity to antimicrobial peptides may be mediated by surface properties, the sensitivity of BT119 to SDS, lysozyme and the antibiotics erythromycin and rifampicin was assayed, but not statistically significant differences were found with respect to the wild-type strain (data not shown).

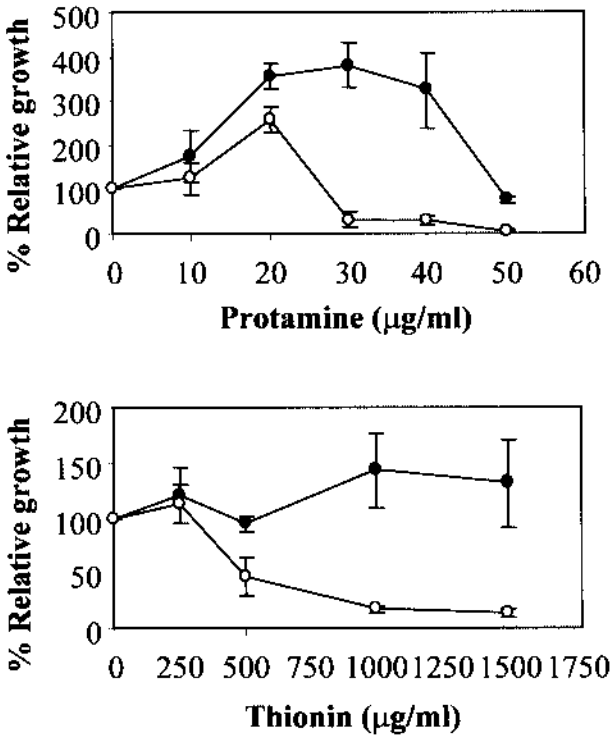

Fig. 2. Susceptibility of *E. chrysanthemi* wild-type (closed circles) and *phoQ* mutant (open circles) to antimicrobial peptides protamine and thionin. Relative growth is expressed as the percentage of O.D. attained by the bacteria after 6 h in the presence of the indicated peptide concentration with respect to the O.D attained in the absence of the peptide. Results show the mean and standard error of three replicates.

The sensitivity of BT119 to acid pH was assayed by a lethality test, performed as indicated in *Experimental procedures*. Figure 3 shows that the *phoQ* mutant had a survival rate about one order of magnitude lower than the wild-type strain at pH 5.5.

Virulence and survival of the phoP-phoQ *mutants in plant tissues*

To investigate the possible effect on virulence of the *phoQ* and *phoP* mutations, assays were performed in witloof chicory leaves and pear fruits. Chicory leaves were inoculated with *E. chrysanthemi* EC16, BT119 and BT118 strains. Necrotic areas of the developed lesions were measured after 48 h, and statistically significant differences were found between the wild-type and BT118 strains, and between wild-type and BT119 strains (Table 2). Similarly, pear fruits were inoculated with *E. chrysanthemi* EC16 and BT119 strains. Necrotic areas of the developed lesions were measured 48 h after inoculation, and statistically significant differences were found between the mutant and the wild-type strains (Table 2).

The relative ability to survive in plant tissues was studied in three plant hosts with different apoplastic pHs, namely, rhubarb stems (pH 3.5), tangerine fruits (pH 4.5) and chicory leaves (pH 5.5). The first two plant tissues are particularly rich in organic acids (Belitz and Grosch, 1985). Plants were inoculated with 10^4 cfu of *E. chrysanthemi* EC16 and the *phoQ* mutant strains. Bacterial populations were estimated at 30 min after inoculation, and results are shown in Fig. 4A. Statistically significant differ-

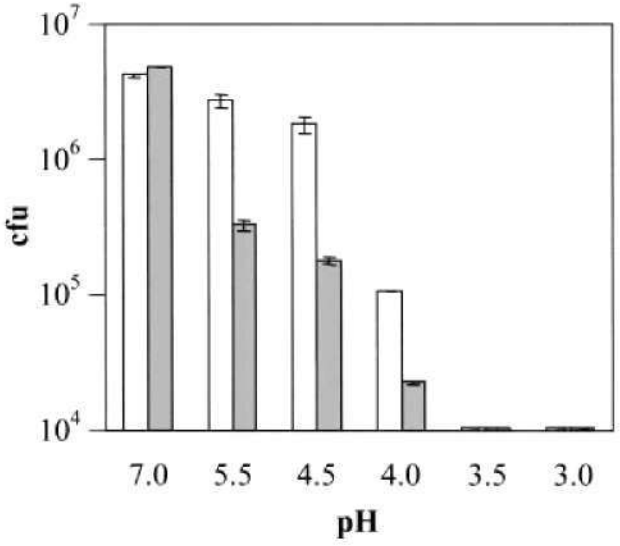

Fig. 3. Survival at different pHs of *E. chrysanthemi* wild-type (empty bars) and *phoQ* mutant (shadowed bars). 10^8 cfu of each strain were incubated 4 h at the indicated pH at 28°C and plated at the appropriate dilution in NB agar plates. Results show the mean and standard error of three replicates.

Table 2. Effects of Δ(*phoQ*)::Tn*5* and Δ(*phoP*)::Tn*7* mutations on the virulence of *E. chrysanthemi* on witloof chicory leaves and pears.

Strain	Size of lesion (cm^2, mean ± SE)[a]
Chicory leaves	
EC16 (wild-type)	0.50 ± 0.06
BT119 (*phoQ*⁻)	0.34 ± 0.02
EC16 (wild type)	0.53 ± 0.06
BT118 (*phoP*⁻)	0.45 ± 0.06
Pears	
EC16 (wild type)	2.08 ± 0.13
BT119 (*phoQ*⁻)	1.46 ± 0.12

a. Values are the product of the length and width of the necrotic area. In all cases, differences between parental and mutant strains were significant according to the Student's test ($P < 0.05$).

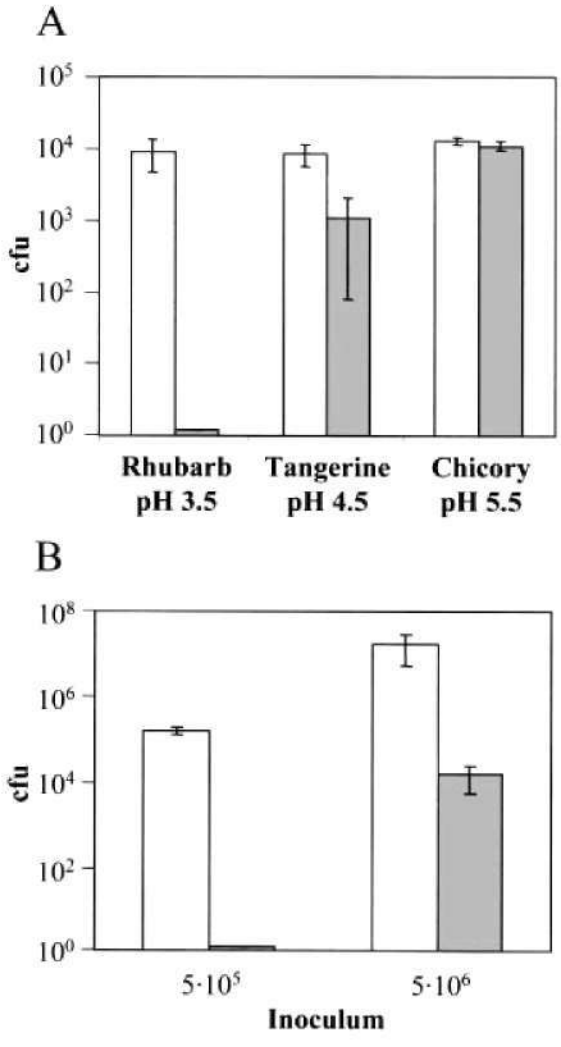

Fig. 4. A. Survival of *E. chrysanthemi* wild-type (empty bars) and *phoQ* mutant (shadowed bars) in rhubarb discs, tangerine segments and chicory discs inoculated with 10^4 cfu, after 30 min of incubation. B. Survival of *E. chrysanthemi* wild-type (empty bars) and *phoQ* mutant (shadowed bars) in rhubarb discs inoculated with 10^5 and 10^6 cfu, after 30 min of incubation.

ences were found in rhubarb and tangerine, but not in chicory. These results suggest that apoplastic pH is an important factor for the survival of the *phoQ* mutant but not for the wild-type strain.

The effect of the inoculum level was studied in rhubarb stems in a similar experiment as the above described. 10^5 and 10^6 cfu of the wild type and the mutant strains were inoculated. As shown in the Fig. 4B the survival of the *phoQ* mutant increased at higher levels of inoculum.

Polygalacturonase production is affected in the phoP *and* phoQ *mutants*

To investigate the possible role of the *phoP-phoQ* operon in the production of extracellular pectic enzymes, polygalacturonase and pectate lyase activities were measured in culture filtrates obtained from *E. chrysanthemi* wild type, *phoP* and *phoQ* mutants. As shown in Table 3, polygalacturonase activity was reduced in the *phoP* mutant (BT118), and almost non-existent in the *phoQ* mutant (BT119). In contrast, the pectate lyase activity was not affected in the mutants (Table 3). Furthermore, the polygalacturonase and pectate lyase activities were assayed *in planta*. Interestingly, the *phoP* and *phoQ* mutants showed a 50% reduction of the total pectate lyase activity (Table 3). In contrast, polygalacturonase activity was undetectable in this type of assay.

As it has been reported that the pH value of the intercellular environment changes as a consequence of *E. chrysanthemi* colonization (Nachin and Barras, 2000). It was decided to investigate if this characteristic is affected in BT119 (*phoQ*⁻). Chicory leaves were inoculated with the wild type and *phoQ* mutant strains; pH indicator solutions were added at the inoculation site in a time-course experiment, and it was observed that the mutant strain lagged behind the wild type in its ability to modify the apoplastic pH around the infection site (Fig. 5).

Table 3. Effects of *Δ*(*phoQ*)::Tn*5* and *Δ*(*phoP*)::Tn*7* mutations on the polygalacturonase activity and pectate lyase activity of *E. chrysanthemi.*

Strain	O.D	In culture polygalacturonase relative activity[a](%)	In culture pectate lyase relative activity[a] (%)
EC16	1.14	100	100
BT118 (*phoP*⁻)	1.16	32.4	102
BT119 (*phoQ*⁻)	1.14	13.5	93

Strain	In planta polygalacturonase relative activity[a](%)	In planta pectate lyase relative activity[a] (%)
EC16	ND	100
BT118 (phoP⁻)	ND	41.6
BT119 (phoQ⁻)	ND	47.6

a. Relative enzymatic activity is expressed as the percentage of activity attained in the spectrophotometric assay respect to that of the wild-type strain. Results show the mean of three replicates. ND, not detected.

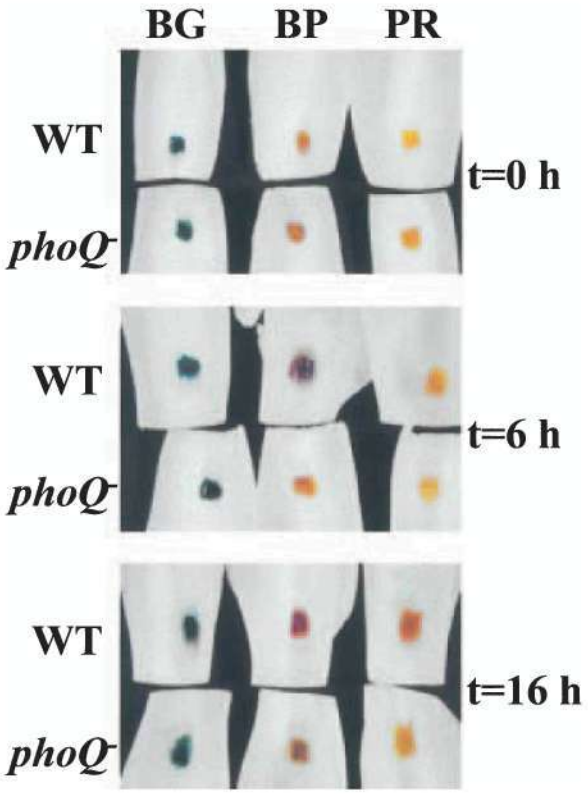

Fig. 5. Time-course of the pH modification of the chicory apoplast shown by colour changes of pH indicator solutions after inoculation with *E. chrysanthemi* wild type and *phoQ* mutant. BG: bromocresol green (3.8–5.4); BP: bromocresol purple (5.2–6.8); PR: phenol red (6.4–8.2).

Regulation of the phoQ *gene in* E. chrysanthemi

The level of expression of the *phoQ* gene was investigated under several conditions by measuring the GUS activity of BT119, taking advantage of the promoterless *uidA* gene contained in the minitransposon (Wilson *et al.*, 1992). To measure GUS activity *in planta*, 0.5 cm diameter chicory discs were inoculated with 10 μl of a suspension containing 10^5 cfu, and were incubated at 28°C for 24 h. Then the discs were homogenized and GUS activity in the homogenate was measured as described in *Experimental procedures*, and the bacterial populations were estimated by dilution plating. A parallel experiment was performed by inoculating 10^5 cfu in 100 μl of NB liquid medium, incubated at 28°C for 24 h, and GUS activity and bacterial populations were estimated as in the previous

case. Similarly, GUS activity was measured in low [Mg^{2-}], low [Ca^{2+}], acid pH and in the presence of sub lethal concentrations of antimicrobial peptides.

As the BT119 mutant was not able to grow at pH 4.5, the estimation of GUS activity at low pH was carried out using a *phoQ* complemented strain (BT121), which contained the entire *phoP-phoQ* operon in a unique-copy vector (Table 1).

Figure 6 shows that the expression of *phoQ* was highly induced *in planta* and moderately induced in low [Mg^{2-}], but not in low [Ca^{2+}], whereas it was unaffected by either acid pH or antimicrobial peptides (data not shown).

Discussion

Only a small number of bacterial genera have evolved as plant pathogens, which is in contrast with the high number of pathogenic bacteria affecting animals. One of the most conspicuous differences between the two types of interaction is that plants represent an acidic milieu, whereas animals offer a slightly alkaline one. *Erwinia chrysanthemi*, like other enteric bacteria, preferably grows at neutral pH (Bearson *et al.*, 1997); which implies that this bacterium is able to colonize the plant apoplast because it has evolved a successful strategy for survival at acid pH. Our observations indicated that strain EC16 of this bacterium was able to grow above pH 4.0, whereas BT119 was unable to grow at pH 5.5, while growing normally at neutral pH. The mutation had pleiotropic effects: (i) diminished virulence in several hosts; (ii) decreased survival ability at acid pH and in plant tissues; (iii) increased susceptibility to antimicrobial peptides from plant and animal origin; and (iv) greatly reduced secretion of polygalacturonases, although normal levels of pectate lyases were observed. The pleiotropic nature of this mutation suggests a regulatory role for the gene affected, which is in line with the high sequence similarity between the mutated gene in BT119 and the *phoQ* gene, which is part of a regulatory system in several bacteria. Indeed, the *phoP-phoQ* operon has been described as a key factor controlling virulence in *Salmonella typhimurium* (Groisman *et al.*, 1989; Miller *et al.*, 1989). PhoQ is a sensor histidine kinase that autophosphorylates in response to environmental conditions and PhoP is a transcriptional regulator, which controls the expression of genes essential for virulence, particularly involved in survival within macrophages, survival at acid pH and resistance to antimicrobial peptides (Fields *et al.*, 1989). The PhoP-PhoQ system also controls the expression of a set of around 40 proteins including proteases, phosphatases and cation transporters (Ernst *et al.*, 2001). Systems of the PhoP-PhoQ type have been identified in a large number of bacterial species; including *Escherichia coli* (Kasahara *et al.*, 1992), *Pseudomonas aeruginosa* (Macfarlane *et al.*, 2000) and *Neisseria meningitidis*

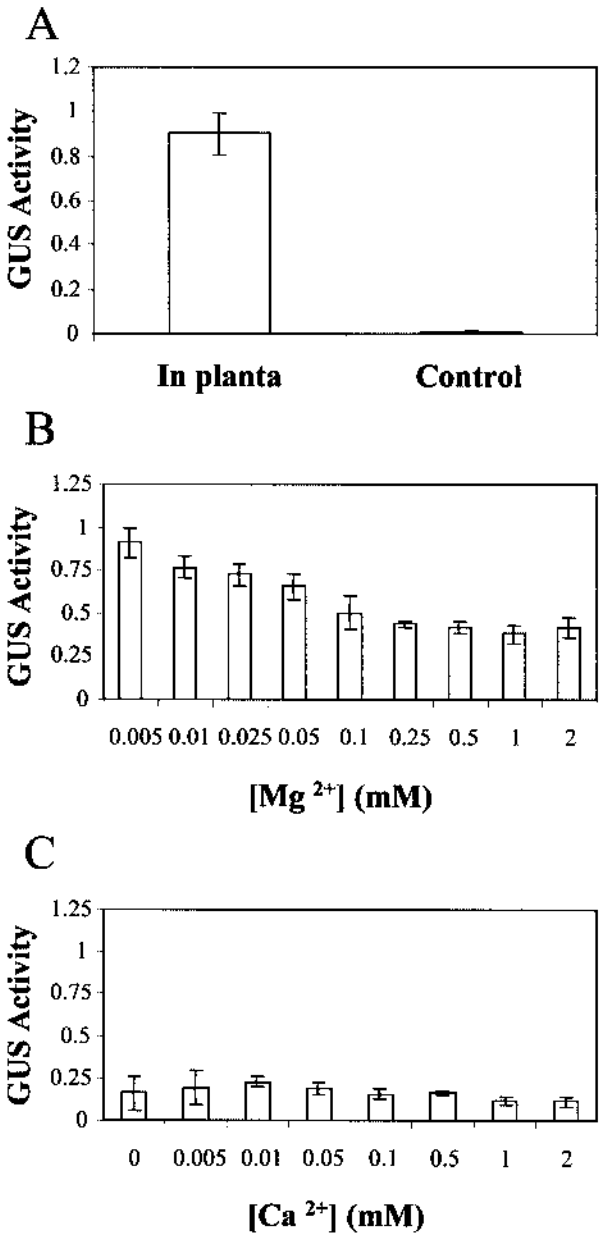

Fig. 6. GUS activity of a *phoQ::uidA* fusion in:
A. Chicory leaves and liquid NB culture.
B. MBMA with the indicated concentrations of Mg^{2-}.
C. MBMA with the indicated concentrations of Ca^{2-}.
Results are expressed as nmoles of *p*-nitrophenol released by 10^6 cells per min 24 h after inoculation. Results show the mean and standard error of three replicates.

(Johnson *et al.*, 2001). The PehR-PehS system, reported by Flego *et al.* (2000) in the phytopathogenic bacteria *Erwinia carotovora*, has some common features with PhoP-PhoQ of *E. chrysanthemi*, as the corresponding mutant is reduced in virulence and lacks polygalacturonase activity. However, it seems that there are certain differences between the *E. carotovora* and E. *chrysanthemi* mutants, as growth at acid pH is not affected in the

former and no differences concerning sensitivity to antimicrobial peptides or survival in plant tissues have been ascribed to it (Flego *et al.*, 2000).

Increased sensitivity toward antimicrobial peptides of BT119 (*phoQ*$^-$) is also seen in the *phoQ* mutant of *S. typhimurium*. It is generally agreed that antimicrobial peptides are an important component of innate immunity in plants, insects, amphibians and mammals (Titarenko *et al.*, 1997; Dimarcq *et al.*, 1998; López-Solanilla *et al.*, 1998; Simmaco *et al.*, 1998; Hancock and Diamond, 2000; García-Olmedo *et al.*, 2001). In the *S. typhimurium* mutant the increased peptide sensitivity is due to the effect of genes regulated by the PhoP-PhoQ system, namely, *pmrA-pmrB* (Gunn and Miller, 1996; Gunn *et al.*, 1998) and *pagP* (Guo *et al.*, 1998). In both cases, the gene product modifies the surface properties of the bacterium by changing the structure of the lipid A in the bacterial lipopolysaccharide. *pmrA-pmrB* are responsible for adding one unit of 4-amino arabinose and *pagP* adds one extra acyl group to LPS. It is tempting to think that a similar mechanism is operating in *E. chrysanthemi*, as genes with high similarity to *pmrA-pmrB* and *pagP* are found in *E. chrysanthemi's* genome (http://www.tigr.org). However, we did not find any difference between the BT119 strain and the wild type with respect to sensitivity to SDS, lysozyme, erythromycin and rifampicin, which suggests that the mutant is not altered in its surface properties. Nevertheless, these experiments were done with bacteria culture in NB medium; thus, we can not rule out that a pathogenicity-activated mechanism may occur, leading to bacterial changes inside the host, as the case of the above mentioned *pagP* gene of *Salmonella typhimurium*.

Our results indicate that the *phoQ* mutant has a more extreme phenotype, as it was less virulent and produced less polygalacturonase than the *phoP* mutant. This is puzzling if the evidence concerning *S. typhimurium* is considered; PhoQ is a sensor kinase which phosphorylates PhoP under the right stimulus; and in turn, the activated PhoP regulates the expression of certain genes. The identification of genes activated or repressed by this system in *Erwinia chrysanthemi*, as well as the detailed mechanism of its regulation, merit future investigations.

Our results clearly indicated that the BT119 strain had statistically significant, albeit small, reduction in virulence on chicory leaves and pear fruits (approximately 70% of wild-type level). However, there is ample experimental evidence showing that *E. chrysanthemi* pathogenicity has several components, and the inactivation of a single component usually produces a slight or even null reduction in virulence. For example, a mutant in the *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) cluster, which is considered of paramount importance in bacterial pathogenicity in plants, retains 75% of wild-type virulence in chicory leaves (López-Solanilla *et al.*, 2001).

In addition to the reduced virulence of BT119 strain, it should be noted its reduced ability to survive in a moderately acid pH and in plant tissues. The difference is drastic (more than one order of magnitude) at pH 5.5, 4.5 and 4.0, which are within the normal range in plant tissues (see Fig. 3). This result is in line with the survival of the mutant strain in plant tissues with different apoplastic pHs (Fig. 4A), where the survival of the *phoQ* mutant increased at higher pHs. Actually, the survival ratio with respect to the wild type was 1.3×10^{-4} at pH 3.5 (rhubarb), 0.12 at pH 4.5 (tangerine) and 0.85 at pH 5.5 (chicory). Interestingly, the differential survival rate in rhubarb stems is dependent on the bacterial load, being less pronounced at high inoculum (see Fig. 4B). A possible explanation of this fact could be related with the ability of this bacterium to change the apoplastic pH in the course of infection (see below). Clearly, the general ability of a bacterium to produce disease in plants depends not only on virulence *per se*, e.g. the ability to colonize a plant tissue, but also on the capacity to survive to several stresses found in nature. This is particularly important in *E. chrysanthemi*, which is able to produce systemic latent infections that become active when conditions favour disease (Perombelon and Kelman, 1980).

The regulation of a virulence regulator such as the PhoP-PhoQ system may have important biological effects. This subject has been studied, using as reporter the glucuronidase (GUS) activity of the strain BT119, which harbours a *uidA* fusion within the structural region of *phoQ*. This gene responds neither to acid pH nor sublethal concentrations of antimicrobial peptides (data not shown). This is in line with observations in other bacteria, where such effects have been ascribed to the indirect action of other genes regulated by the PhoP-PhoQ system (García *et al.*, 1996). However, GUS activity was enhanced *in planta* and at low magnesium but not at low calcium concentrations (see Fig. 6). The effect of low [Mg^{2+}] has been reported in *Salmonella*, where it was interpreted as part of a regulatory loop that increases the effect of the system when the bacterium enters the mammalian host, an environment that is particularly low [Mg^{2+}] (García *et al.*, 1996). In the case of *E. chrysanthemi*, it is difficult to ascertain the actual [Mg^{2+}] surrounding the bacterium in the apoplast, although it has been hypothesized that such concentration is low in the first stages of infection, as magnesium, contrary to calcium, is not abundant in plant cell walls (Emanuele and Staples, 1990). Presently, we do not have an explanation for the observed increased of GUS activity *in planta*. However, several hypothesis can be formulated: (i) the PhoP-PhoQ system may be positively controlled by chemical signals from the plant; (ii) the concentration of several nutrients (as we have seen for

magnesium) may have an influence in the regulation of *phoP-phoQ*; and (iii) the system could be under the control of other regulatory networks. Further work will be needed to contrast these hypothesis.

When *E. chrysanthemi* colonizes the plant extracellular space, it secretes several pectolytic enzymes which degrade the plant cell wall, inducing cell lysis. The liberation of cell contents to the apoplast produces the alkalinization of the space surrounding the bacteria. This, in turn, affects the activity of the different pectic enzymes and, also, the expression of the corresponding genes (Nachin and Barras, 2000). It should be noted that *E. chrysanthemi* secretes two major types of pectic enzymes: polygalacturonases, which cleave by hydrolysis, and pectate lyases, which cleave by β-elimination (Collmer and Keen, 1986). The former are thought to be less important, and in fact, mutants with a deletion in the *pehVWX* cluster, which is responsible for most of the polygalacturonase activity, showed virtually full maceration capacity (Nasser *et al.*, 1999). However, polygalacturonases have optimal activity at acidic pHs whereas most pectate lyases work better in alkaline conditions (Collmer and Keen, 1986). Therefore, polygalacturonases may have a crucial role in changing the apoplastic pH in the early phase of the infection, facilitating the further action of pectate lyases (He and Collmer, 1990). This hypothesis is in line with our finding that the mutant BT119 changes the extracellular pH slower than the wild type (see Fig. 5). However, we found that the pectate lyase activity of the mutants are diminished *in planta* but not in culture. A possible explanation relies on the fact that *E. chrysanthemi* EC16 possess several *in planta*-inducible pectate lyases (Kelemu and Collmer, 1993), which expression could be affected in the *phoP* and *phoQ* mutants.

In conclusion, our results support the indicate that the PhoP-PhoQ system of *E. chrysanthemi* controls a set of characteristics that act co-ordinately to help the establishment and survival of bacterial population in the plant tissue, namely, the abilities to grow at acid pH, to alkalinize the external pH, and to withstand antimicrobial peptides. These features seem to be far more important at the low inocula prevailing in natural infections, and may play an important role in adapting the bacterium to the changing conditions found along its life cycle. Also, these results imply that the PhoP-PhoQ regulatory system has become adapted to counteract different but related stresses in plant and animal pathogenic bacteria.

Experimental procedures

Microbiological methods

The bacterial strains and plasmids used in this study are described in Table 1. Strains of *Escherichia coli* were cultivated at 37°C in Luria–Bertani medium. Strains of *E. chrysanthemi* were cultivated at 28°C in nutrient broth (NB; Difco), King's B medium (King *et al.*, 1954) or modified basal medium A (MBMA) (Torriani, 1960) (citric acid monohydrate was added instead of sodium citrate to buffer in the lower pH ranges (Vogel and Bonner, 1956; Foster and Hall, 1990) supplemented with 0.2% glycerol-250 μM potassium phosphate (pH 7.0) (Roeder and Collmer, 1985). Antibiotics were added to the media at the following concentrations (μg ml^{-1}): ampicillin, 100; spectinomycin, 50; streptomycin, 10; kanamycin, 20; and nalidixic acid 20. Plasmid pCAM140 containing a minitransposon with the promoterless reporter gene GUS (Wilson *et al.*, 1995) was used for random mutagenesis of the bacterial genome.

DNA manipulation and sequencing

A genomic library of *E. chrysanthemi* was constructed in the λ-FIX II (Stratagene). Plasmid pBluescript SK(–) (Stratagene) was used for subcloning. Tn*7 in vitro* mutagenesis was performed with the Genome Priming System (GPS-1) kit (New England Biolabs). Marker exchange in *E. chrysanthemi* was performed as described (Roeder and Collmer, 1985). Standard molecular cloning techniques employed in this study were performed as described previously (Sambrook *et al.*, 1989). DNA sequencing of both strands was done by the chain termination method on double-stranded DNA templates with an ABI Prism Dye Terminator cycle sequencing kit (Perkin-Elmer) in a 377 DNA Sequencer (Perkin-Elmer). Sequence alignments were performed at the National Center for Biotechnology Information (on-line) with the BLAST network service (Altschul *et al.*, 1997).

Susceptibility, lethality and virulence assays

Susceptibility to antimicrobial peptides was assayed as previously described (López-Solanilla *et al.*, 1998). To perform lethality assays, 10 ml of MBMA at pH = 3.0, 3.5, 4.0, 4.5, 5.5 and 7.0, were inoculated with 100 μl of a suspension containing 10^6 bacterial cells of wild-type or *phoQ* mutant strains. Cells were incubated for 4 h at 28°C with shaking, and then a portion of each sample was diluted and plated on nutrient broth agar plates to assess bacterial viability. Three replicates were performed in each case.

Susceptibility to erythromycin, lysozyme and rifampicin were assayed as previously described (López-Solanilla *et al.*, 1998). The SDS sensitivity assays were performed by seeding NB top agar with 100 μl aliquots of stationary-phase cultures of the wild-type and *phoQ* mutant strains. After solidification, discs containing different SDS concentrations were placed on the medium and incubated at 28°C for 24 h.

Virulence or survival assays were performed in witloof chicory leaves, pears, rhubarb (*Rheum rhabarbarum* L) and tangerine purchased from a local supermarket. The cells from an overnight NB liquid medium culture were washed with 10 mM $MgCl_2$ and then resuspended in an appropriate volume of buffer to obtain the desired inoculum concentration. Virulence assays on chicory leaves were performed as previously described (Bauer *et al.*, 1994). Briefly, each chicory leaf was inoculated at two locations with 10 μl of a suspension containing 10^5 bacterial cells, and 35 leaves were pair-inoculated with the wild-type and each mutant strains.

Chicory leaves were incubated for 48 h in a moist chamber at 28°C. The macerated area was measured and differences between wild-type and mutants strains were statistically assessed with a paired Student's *t*-test. To monitor pH variations in chicory leaves upon infection, the following pH indicator solutions (0.1%) were prepared: (i) bromocresol green (Merck), which is yellow at a pH value below 3.8 and blue at a pH value above 5.4; (ii) bromocresol purple (Merck), which is yellow at a pH value below 5.2 and purple at a pH value above 6.8, and; (iii) phenol red (Merck), which is yellow at a pH value below 6.4 and red-violet at a pH value above 8.2. The chicory leaves were incubated in a moist chamber at 28°C and were analysed at different times by adding the pH indicator solutions at the inoculation site.

Pears were inoculated with 50 µl of a suspension containing 5×10^5 bacteria by inserting a plastic micropipettor tip at a constant depth of 1.5 cm. The experiment was performed using 40 pears. Each fruit was inoculated separately at different points in the same fruit with wild-type and *phoQ* mutant strains, in order to minimize the effect of the variability among individual pears. Pears were left at 28°C, 100% relative humidity for 48 h. After this time, fruits were sliced at the inoculation point and the damage was estimated by measuring the macerated area. Differences between the wild-type and mutant strains were statistically assessed with a paired Student's *t*-test.

For survival assays in plant tissues, 1 cm of diameter of rhubarb and chicory discs, and tangerine segments were used. Plant tissues were inoculated with 10^4 bacterial cells of the wild-type and *phoQ* mutant strains and incubated at 28°C and high humidity. Bacterial populations were estimated after 30 min by tissue homogenization in an appropriate volume of 10 mM $MgCl_2$, followed by dilution plating. Three replicates were used to calculate mean and standard error.

Enzymatic assays

Culture filtrates containing a mixture of extracellular pectic enzymes were obtained as previously described (Miguel *et al.*, 2000). For the determination *in planta* of the enzymatic activities, chicory discs were inoculated with a suspension containing 10^7 bacterial cells, and incubated at 28°C. After 4 h, the tissues were treated as indicated for the culture filtrates. The polygalacturonase (Peh) activity was determined by measuring the release of reducing groups in culture. The extracellular pectate lyase (Pel) activity was determined by monitoring the increase of absorbance at 232 nm as a result of the 4,5-unsaturated reaction products. Both assays were performed as described by Collmer *et al.* (1988). Glucuronidase activity was measured by the spectrophotometric assay, described by Wilson *et al.* (1992).

Nucleotide sequence accession numbers

The DNA sequences determined in this study were deposited in the DDBJ/EMBL/GenBank nucleotide sequence database under the accession number AJ489252 (*phoP* and *phoQ* genes).

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